Kidney International, Vol. 61 (2002), pp. 2025–2032

CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

Inhibiting albumin glycation in vivo ameliorates glomerular overexpression of TGF-β1

Margo P. Cohen, Fuad N. Ziyadeh, Soon Won Hong, Clyde W. Shearman, Elizabeth Hud, Gregory T. Lautenslager, M. Carmen Iglesias-de la Cruz, and Sheldon Chen

Institute of Metabolic Research and Exocell, University City Science Center, and Renal-Electrolyte and Hypertension Division, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Inhibiting albumin glycation in vivo ameliorates glomerular overexpression of TGF-β1.

Background. Glycated albumin has been causally linked to the pathobiology of diabetic renal disease through its ability to stimulate the expression of transforming growth factor-β1 (TGF-β1), activate protein kinase C (PKC) and extracellular signal-regulated kinase (ERK), and promote production of extracellular matrix proteins in cultured glomerular cells. Whether glycated albumin modulates glomerular TGF-β1 expression in vivo is not known. To address this issue, we assessed glomerular TGF-β1 expression and pathology in response to reducing the burden of glycated albumin in vivo.

Methods. We measured serum glycated albumin, urine protein, glomerular TGF-β1 expression and morphometry, and collagen IV and fibronectin mRNA in *db/m* and *db/db* controls and in *db/db* mice treated for eight weeks with a synthetic compound that inhibits the condensation of glucose with albumin.

Results. In situ hybridization studies showed markedly increased glomerular TGF-β1 mRNA in control *db/db* mice, which was significantly reduced in *db/db* mice treated for eight weeks with test compound. The treatment protocol, which normalized serum glycated albumin, concomitantly reduced the elevated protein excretion and the renal overexpression of mRNAs encoding fibronectin and collagen IV, and significantly decreased the mesangial matrix expansion, observed in *db/db* control animals.

Conclusions. These findings, to our knowledge, provide the first evidence that glomerular overexpression of TGF-β1 in diabetes derives at least in part from elevated glycated albumin concentrations, and can be partially suppressed by inhibiting the formation of this glycated protein. The results further suggest that glycated albumin has an important nephropathogenic role in diabetes that is operative, and can be therapeutically addressed, independent of glycemic status.

Key words: diabetic nephropathy, transforming growth factor-β1, glycated albumin, *db/db* mouse, hyperglycemia.

Received for publication October 8, 2001
and in revised form January 7, 2002
Accepted for publication January 8, 2002

Hyperglycemia and the consequent accelerated nonenzymatic glycation of proteins are important risk factors in diabetic nephropathy [1–3]. In diabetes, serum entering the glomerular capillary bed contains increased concentrations of albumin modified by Amadori glucose adducts, the principal circulating glycated protein [2–5]. In renal glomerular mesangial and endothelial cells, Amadori-modified glycated albumin stimulates the expression of the fibrogenic transforming growth factor-β1 (TGF-β1) and its primary signaling receptor, the TGF-β type II receptor, activates protein kinase C (PKC) and extracellular signal-regulated kinase (ERK), and increases production of the extracellular matrix (EMC) proteins fibronectin and type IV collagen [6–13]. These effects on ECM proteins synthesis and cytokine expression and activity are observed in physiologic (5.5. mmol/L) glucose concentration with concentrations of glycated albumin that are found in clinical specimens, and are not observed with the nonglycated counterpart. Additionally, glycated albumin can stimulate H_2O_2 production (abstracts; Aggarwal et al, *Clin Res* 40:179A, 1992; Daniels et al, *J Am Soc Nephrol* 4:791A, 1993), promote glomerular hyperfiltration [14], and increase the expression of nitric oxide synthase and the production of nitric oxide [15]. The concentration of Amadori-modified albumin is independently associated with diabetic nephropathy [16], and localization of the glycated protein in glomeruli of patients with diabetic nephropathy corresponds with the severity of renal involvement [17]. These studies have provided evidence that increased glycated albumin is an important contributor to the development of diabetic nephropathy, an interpretation that is supported by experimental results showing that treatment of diabetic *db/db* mice with monoclonal antibodies that neutralize deoxyfructosyllysine epitopes in glycated albumin ameliorates the characteristic renal structural and functional abnormalities [18–20].

Although the in vitro studies summarized above sug-

gest that increased glycated albumin helps promote the glomerular overexpression of TGF-β that is observed in diabetes [21–23], direct evidence supporting this relationship in vivo is lacking. To examine this hypothesis, we assessed glomerular TGF-β1 expression in *db/db* mice after treatment with a compound that impedes the condensation of glucose with albumin. We report that reducing the burden of glycated albumin by inhibiting its formation, even in the presence of hyperglycemia, significantly decreases glomerular TGF-β1 expression and associated pathobiologic abnormalities. To our knowledge, this is the first in vivo study demonstrating a causal link between glycated albumin and TGF-β1-stimulated glomerulosclerosis.

METHODS

Experimental animals

The studies were performed in male diabetic *db/db* mice and age-matched male nondiabetic *db/m* mice (Jackson Laboratory, Bar Harbor, ME, USA) that were provided food and water ad libitum. At baseline, midpoint and completion of the experimental protocol, blood was obtained from the retro-orbital sinus for measurement of glucose and glycated albumin and 24-hour urines were collected for measurement of albumin excretion.

Treatment protocol

Diabetic mice were divided into two groups of six, one of which received test compound and the other serving as the diabetic control. Compound was administered by gavage for eight consecutive weeks at a dose of 10 mg/kg body wt/day, commencing when animals were 8 weeks old. Control diabetic and nondiabetic mice received daily saline gavage for the same 8-week period. At the conclusion of the treatment period, one kidney from each animal was harvested for RNA extraction from renal cortex (separated by gross dissection), with a portion of the snap frozen specimen reserved for in situ hybridization studies. The other kidney was fixed by immersion in 10% neutral buffered formalin and embedded in paraffin for histologic examination.

Preparation and analysis of test compound

The compound (23CPPA) was synthesized via condensation of 2-bromophenylacetic acid with a chloro-substituted aniline and purified through filtration, organic extraction and crystallization by described techniques [24]. It migrated as a single spot on silica gel thin layer chromatography, exhibited appropriate aromatic and acid protons on nuclear magnetic resonance (NMR), and had the correct composition on elemental analysis ($C_{14}H_{12}ClNO_2$), with a molecular weight of 261.7 and a melting point of 102°C. This compound is in the same structural class as the anti-inflammatory agent diclofenac, which has been shown to bind to well-characterized sites in serum albumin and render susceptible ε-amino groups inaccessible for condensation with free reducing sugar, but lacks the 2,6 dichloro substitution required for inhibition of cyclooxygenase (COX) [24–26]. We confirmed low COX inhibitory activity by incubating COX-1 purified from ram seminal vesicles in the presence of cofactors, substrate, and 0 to 1000 μmol/L of compound, and measuring the peroxidase activity according to the vendor's instructions (Cayman Chem Co., Ann Arbor, MI, USA). The IC_{50} for COX inhibition of 23CPPA was >600 μmol/L, compared to an IC_{50} of 1 μmol/L for diclofenac assayed by the same procedure. The ability of the compound to inhibit the nonenzymatic glycation of albumin in vitro was tested by incubating 0 to 1000 μmol/L of 23CPPA with serum albumin for two days at room temperature in the presence of 50 mmol/L glucose in buffered saline, and determining the amount of glycated albumin formed. This was accomplished by processing the reaction mixtures through Amicon filters to remove free glucose and compound, followed by application to phenylboronate agarose (PBA) to separate nonglycated (unbound) from glycated (bound) albumin. The albumin concentration in the bound fraction, eluted with 0.1 mol/L sorbitol, was measured by immunoassay with anti-human albumin specific antibody [27]. The IC_{50} for inhibition of albumin glycation by test compound in this assay was 1 μmol/L.

Analytical and immunoassay procedures

Glucose was determined by the glucose oxidase method (Sigma, St. Louis, MO, USA). Urine albumin was measured with a competitive enzyme-linked assay that has been described previously [19]. Serum concentrations of glycated albumin were measured after affinity chromatography on PBA as described above, with determination of the albumin concentration in the 0.1 mol/L sorbitol elution by immunoassay specific for mouse albumin.

RNA hybridization analysis

Northern analysis. RNA extracted from snap-frozen renal cortex was prepared as previously described [9, 10, 19].

Total RNA (20 μg from each animal) was electrophoresed on 1.2% agarose gels, transferred onto nylon membranes, and ultraviolet wave cross-linked. Integrity and equal loading of the samples were assessed by methylene blue staining of the transferred RNA. The membranes were hybridized with ^{32}P-labeled cDNA probes encoding fibronectin and α1(IV) collagen as described [9, 10, 19]. After hybridization, blots were washed for ten minutes in 2 × standard sodium citrate (SSC) at room temperature, then for five minutes in 2 × SSC/1% sodium dodecyl sulfate (SDS) at 62°C, then for five minutes in 0.1 × SSC/0.1% SDS at 62°C. The membranes were autoradiographed with intensifying screens at −70°C for up to ten

days. Blots were then stripped for two hours at 62°C with 5 mmol/L Tris, 0.2 mol/L ethylenediaminetetraacetic acid (EDTA; pH 8.0), and 5% sodium pyrophosphate, and subsequently rehybridized with cDNA fragment encoding an RNA standard (mouse ribosomal protein L32; mrpL32) [28]. Exposed films were scanned with a laser densitometer, and mRNA levels were calculated relative to those of the loading standard.

TGF-β1 in situ hybridization. The mouse TGF-β1 cDNA was subcloned into pcDNA 3 vector (Invitrogen) and linearized with *NotI* for antisense and *Hin*dIII for sense orientation [21]. The linearized plasmids were gel isolated, and the transcription reaction for nonisotopic labeling of riboprobe was performed according to the manufacturer's instructions (Boehringer Mannheim). The mixture of digoxigenin labeling (50 μL) included 1 μg template cDNA, 2 μL NTP labeling mixture, 2 μL transcription buffer, 1 μL RNase inhibitor, and 2 μL T7 or SP6 polymerase. Transcription was performed for two hours at 37°C followed by digestion with 2 μL RNase-free DNase for 15 minutes at 37°C. The riboprobe was purified with spin columns. For in situ hybridization, frozen kidney sections (5 μm) were overlaid with 30 μL of hybridization buffer containing the labeled RNA probe and incubated at 58°C overnight in a humid chamber. After hybridization, sections were washed once in 2 × SSC at room temperature for 30 minutes, once in 2 × SSC at 65°C, and once in 0.1 × SSC at 65°C. Slides were equilibrated with buffer I (100 mmol/L Tris HCl/150 mmol/L NaCl, pH 7.5) for five minutes. Anti-digoxigenin antibody conjugated to alkaline phosphatase (1:5000) was applied to the slides and incubated in a humid chamber for two hours at room temperature. After two 15-minute washes in buffer I, the sections were equilibrated with developing buffer (100 mmol/L Tris/100 mmol/L NaCl/50 mmol/L $MgCl_2$, pH 9.5) for five minutes. The color reaction was developed by NBT/BCIP (Boehringer) according to the manufacturer's instructions. Quantitation was performed by computer program IMAGE-PRO PLUS (Media Cybernetics, Silver Spring, MD, USA).

Glomerular pathology

Random sections (3 μm thick) of the renal cortex were stained with periodic acid-Schiff (PAS). Sections were coded and read by an observer unaware of the experimental group from which they were derived. Ten glomeruli in the outer cortex were selected at random from each animal in each experimental group. Mesangial matrix fraction was determined as PAS positive material present in the mesangial region, exercising care to exclude cellular elements. The mesangial matrix area was calculated as the fraction of total glomerular tuft cross-sectional area as previously described [19]. Values obtained with this method were consistent with those reported by other investigators using light and/or electron microscopic methods. Increased mesangial fractional volume is a better indicator of declining filtration function than is width of the glomerular basement membrane [29–32].

Table 1. Experimental animal data

Age	Group	Body wt *g*	Glucose *mmol/L*	Kidney wt *mg*
8 weeks*	*db/db* control	38.7 ± 1.2[a]	18.1 ± 2.0[a]	
	db/db treated	37.8 ± 1.0[a]	17.5 ± 1.8[a]	
	db/m control	20.0 ± 0.5	5.8 ± 0.7	
12 weeks	*db/db* control	37.7 ± 2.0[a]	23.5 ± 2.3[a]	
	db/db treated	35.1 ± 1.0[a]	24.3 ± 2.1[a]	
	db/m control	26.2 ± 0.7	6.0 ± 0.8	
16 weeks	*db/db* control	34.5 ± 3.3[a]	27.8 ± 2.2[a]	465 ± 28[a,b]
	db/db treated	33.6 ± 0.9[a]	26.9 ± 3.0[a]	428 ± 21[a]
	db/m control	27.5 ± 0.4	6.1 ± 0.7	353 ± 10

* Eight week values are baseline (pre-treatment). Groups included: *db/db* and *db/m* control, saline gavage; *db/db* treated, 10 mg/kg/day test compound by gavage; $N = 6$ in each experimental group.
[a] $P < 0.05$ vs. *db/m*
[b] $P < 0.05$ vs. *db/db* treated

RESULTS

Animal characteristics

The *db/db* diabetic mutant mouse develops hyperglycemia associated with obesity and insulin resistance a few weeks after birth, and exhibits pronounced glomerular mesangial expansion by age 16 weeks [18, 19, 33–35]. General characteristics of the experimental animals used in the present study conformed with those known to be associated with this model (Table 1). The diabetic *db/db* mice were obese and hyperglycemic at the start and conclusion of the study. The mean body weight was greater in *db/db* mice compared to *db/m* controls throughout the experimental period, although diabetic animals stopped gaining weight between ages 12 and 16 weeks, consistent with entry into an insulin-deficient catabolic state as reported in this rodent model [34, 35]. The treatment protocol did not affect body weight or blood glucose; since the test compound binds to albumin and does not react with free glucose, no effect on metabolic status was expected. The protocol was well tolerated by the mice and had no obvious toxicity. Necropsy revealed no gross pathology in major organs in the treated mice. Kidney weights at the end of the study were significantly greater in diabetic compared to nondiabetic controls, and the mean kidney weight was significantly less in the *db/db* mice that received test compound than in the *db/db* controls.

Glycated albumin

Serum concentrations of glycated albumin in diabetic animals were ≈40% higher than in nondiabetic mice at the start of the experimental period, reflecting exposure

Table 2. Glycated albumin concentrations

Age	Group	Glycated albumin μg/mL
Baseline	*db/db* control	1142 ± 114[a]
	db/db treated	1312 ± 147[a]
	db/m control	942 ± 30
Midpoint	*db/db* control	1318 ± 145[a]
	db/db treated	1050 ± 51[ab]
	db/m control	909 ± 46
Conclusion	*db/db* control	1511 ± 60[a]
	db/db treated	1129 ± 77[ab]
	db/m control	1038 ± 37

Experimental animal groups are defined in Table 1. Serum glycated albumin concentrations were measured as described in the text.

[a] $P < 0.05$ vs. *db/m* control

[b] $P < 0.05$ vs. *db/db* control

to a hyperglycemic milieu during the preceding one to two weeks [2–4]. Glycated albumin concentrations in diabetic mice receiving test compound were significantly lower than in the corresponding diabetic controls at the midpoint and conclusion of the study (Table 2). This sustained reduction in glycated albumin occurred despite the persistent and marked elevation in blood glucose concentrations in the *db/db* mice treated with test compound.

Glomerular TGF-β1 expression

In situ hybridization studies demonstrated that glomerular TGF-β1 expression was markedly increased in *db/db* mice, consistent with previous observations [21]. Compared to nondiabetic mice, diabetic control mice showed a four- to fivefold selective increase in TGF-β1 mRNA in a mesangiocapillary pattern within the glomerular tuft (Fig. 1 A–D). This increase was reduced by 45% in diabetic animals that received test compound (Fig. 1E) in which the treatment protocol significantly decreased glycated albumin but not glucose concentrations.

Matrix expression and glomerular morphometry

Northern blot analysis indicated that corresponding changes in renal ECM protein expression accompanied the changes in TGF-β1 expression. Steady state levels of mRNAs encoding fibronectin and α1 (IV) collagen were significantly greater in the renal cortex from *db/db* controls compared to nondiabetic *db/m* mice, as previously reported (Fig. 2) [19, 21]. Densitometric scanning of the exposed autoradiographs and calculation of ECM protein mRNA levels in relation to the mrpL32 standard indicated that renal expression of fibronectin was normalized, and expression of α1 (IV) collagen was significantly reduced, in *db/db* mice treated with test compound (Fig. 2).

The increased renal ECM and TGF-β1 expression in *db/db* controls at the conclusion of the study was accompanied by the glomerular histopathologic changes that have been described in this model, including diffuse mesangial expansion and increased mesangial matrix encroaching on the capillary network [18, 19, 21]. Quantitative glomerular morphometry indicated that the mesangial matrix fraction in control *db/db* mice was approximately 3.6 times greater than in *db/m* mice (Fig. 3A). In contrast, the mean mesangial matrix fraction in treated *db/db* mice was only 2.2 times greater than in nondiabetic controls, representing a reduction in mesangial matrix accumulation of more than 40% (Fig. 3A). Glomerular tuft surface area in *db/db* mice was 30% greater than in *db/m* animals, and was unaffected by treatment with test compound (Fig. 3B).

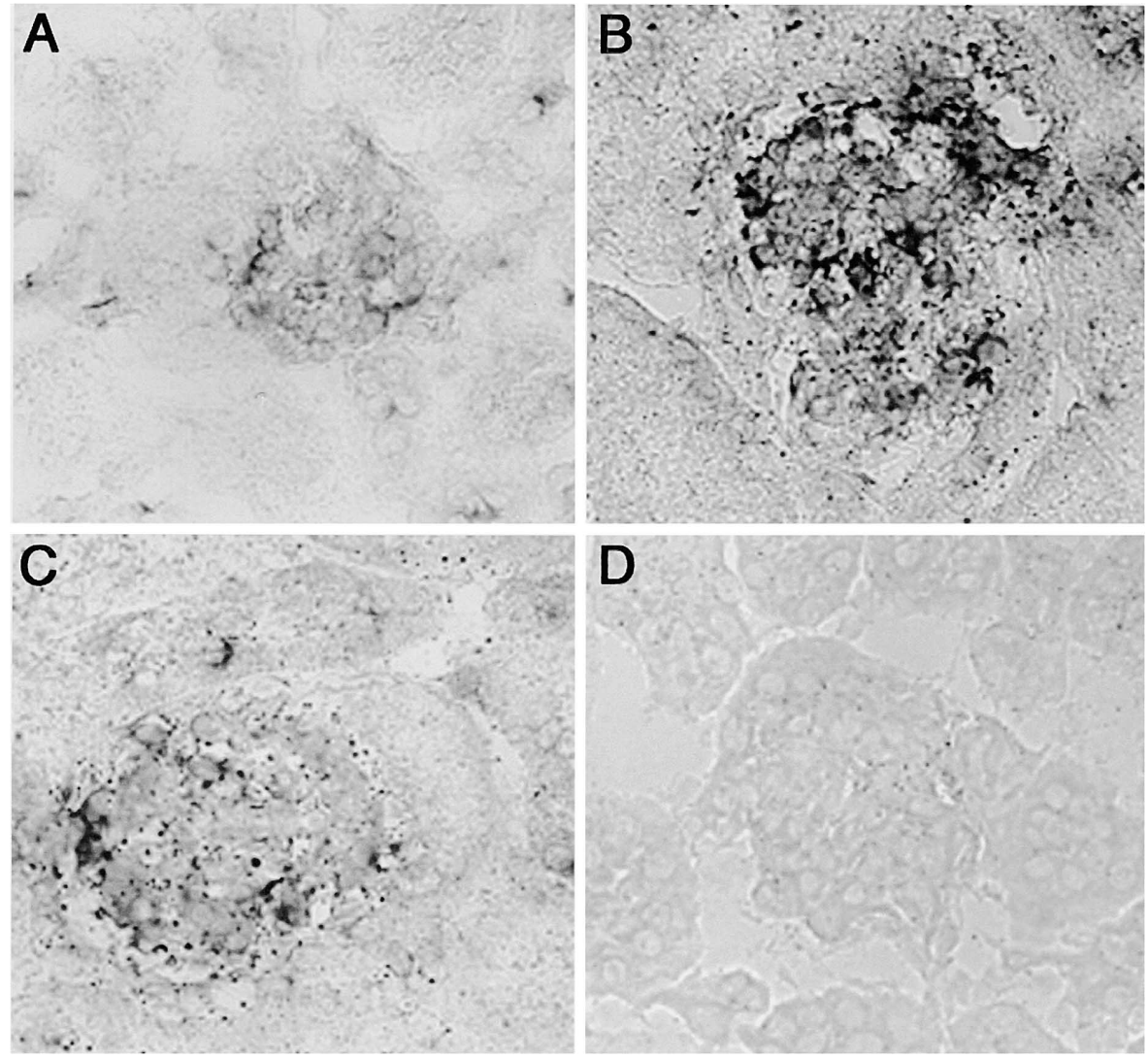
Albumin excretion

Increased albumin excretion, the established parameter of early diabetic renal dysfunction [36, 37], was present in the *db/db* mice by age eight weeks, and albumin excretion remained significantly elevated in *db/db* controls compared to *db/m* mice at levels consistent with those reported by other investigators using similar methodology (Fig. 4) [38]. The *db/db* mice receiving test compound showed a 45% reduction in albumin excretion (Fig. 4). The absence of a significant difference in the mean body weight in treated versus control diabetic mice supports the interpretation that the decrease in albumin excretion was not due to caloric deprivation, although the lack of complete normalization of albumin excretion is consistent with the continued severe hyperglycemia.

DISCUSSION

The results of these studies provide evidence that glomerular overexpression of TGF-β1 in diabetes derives at least in part from the elevated circulating glycated albumin concentrations associated with this disease. Glycated albumin is known to activate the TGF-β system in glomerular mesangial and endothelial cells [9, 12], and treatment with anti-TGF-β antibody has been shown to prevent pathology in the *db/db* mouse [21], suggesting that TGF-β may be the common downstream mediator of deleterious factors promoting glomerular fibrosis in diabetes. The increased glomerular TGF-β1 mRNA in diabetic mice was significantly reduced in conjunction with a lowering of serum glycated albumin concentrations by test compound, consistent with the interpretation that the associated amelioration of matrix protein overexpression and glomerulosclerosis were mediated by this reduction in glomerular TGF-β1.

The treatment protocol also decreased urine albumin excretion in *db/db* mice, a finding of interest in view of the report implicating increased glycated albumin to perm-selective changes in the glomerular basement membrane that underlie albuminuria early in the course of diabetes [39]. The lowering of urinary albumin in association with normalization of serum glycated albu-



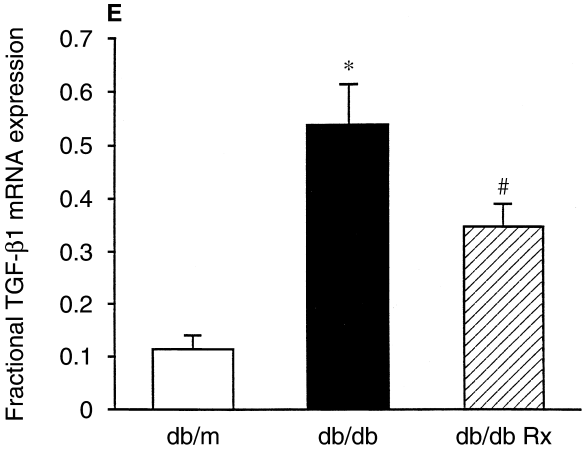


Fig. 1. In situ hybridization with antisense transforming growth factor-β1 (TGF-β1) riboprobe at age 16 weeks in *db/m* (*A*), *db/db* (*B*), and *db/db* mice after 8 weeks of treatment with test compound (*C*). Sense TGF-β1 riboprobe in 16-week-old saline-treated *db/db* mouse showed absence of hybridization signal as negative control (*D*). Quantitation (*E*) was performed as described in the text in each of 4 to 6 animals in each experimental group, and calculated as area of signal (μ^2) divided by area of glomerulus (μ^2). $^*P < 0.05$ vs. *db/m* control; $\#P < 0.05$ vs. *db/db* control.

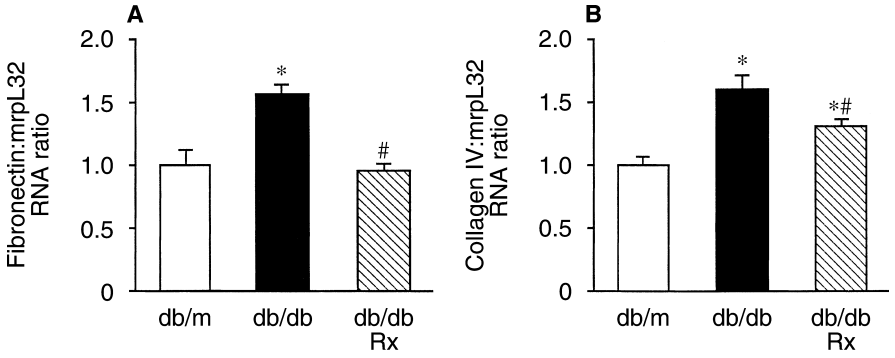


Fig. 2. Renal fibronectin (*A*) and α1 (IV) collagen (*B*) gene expression in experimental animals. Total RNA was extracted from snap-frozen renal cortex when animals were killed at the end of the study period. Fibronectin and α1 (IV) collagen mRNA levels were calculated in relation to mrpL32 after densitometric scanning of the exposed films. Results represent mean ± SEM of 4 to 6 animals in each group when the ratio of fibronectin or collagen IV mRNA to mrpL32 RNA in *db/m* saline-treated mice is assigned an arbitrary value of 1. $*P \leq 0.05$ in *db/db* control (■) vs. *db/m* (□); $\#P \leq 0.05$ in *db/db* treated with test compound (▨) vs. corresponding *db/db* control.

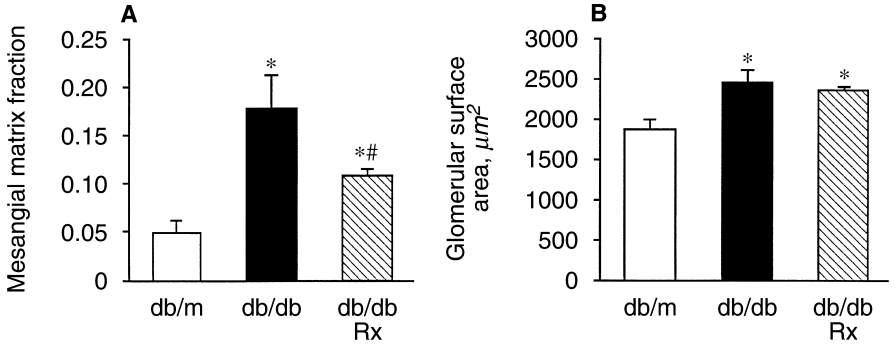


Fig. 3. Mesangial matrix fraction (*A*) and glomerular tuft surface area (*B*) in glomeruli from nondiabetic *db/m* control mice (□) and in diabetic *db/db* mice receiving saline (■) or test compound (▨). $*P \leq 0.05$ vs. nondiabetic *db/m*; $\#P \leq 0.05$ vs. corresponding *db/db* control.

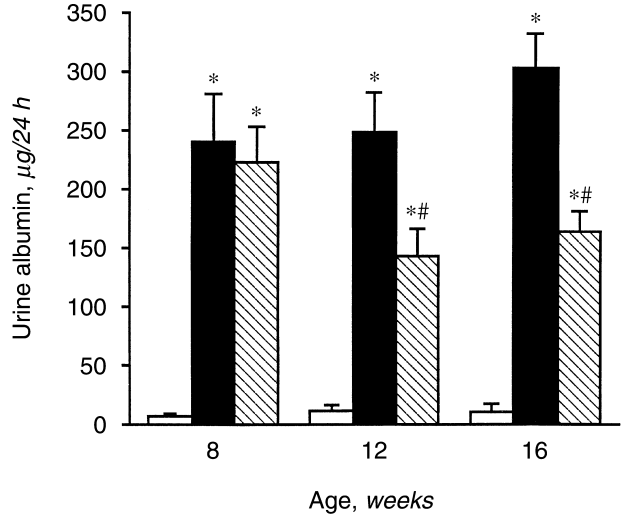


Fig. 4. Urine albumin excretion measured at initiation, midpoint and conclusion of the experimental protocol in nondiabetic *db/m* mice (□) and in diabetic *db/db* mice receiving gavage with saline (■) or test compound (▨). $N = 6$ in each experimental group. $*P \leq 0.05$ vs. *db/m* control at same age; $\#P \leq 0.05$ vs. corresponding *db/db* control at same age.

min lends credence to this hypothesis. It is not clear if a lessening of the stimulation of TGF-β1 by glycated albumin contributed to this reduction in albumin excretion. A recent study found that anti-TGF-β antibody treatment did not affect albuminuria in *db/db* mice [21], prompting the interpretation that TGF-β is not a key factor in the glomerular hemodynamic and permeability changes responsible for proteinuria and/or that proteinuria promotes progression of renal disease through activation of the tubulointerstitial TGF-β system [40, 41]. Whereas our finding that the test compound lowered, but did not normalize, urinary albumin does not negate this interpretation, it does suggest that decreasing glycated albumin may ameliorate proteinuria by TGF-β1–independent mechanisms. Further, the decreased urinary albumin cannot be ascribed to inhibition of COX activity, which can modulate pathological renal hemodynamics contributory to diabetic nephropathy [42], since the test compound has very low affinity for COX enzymes (IC_{50} ≈600 μmol/L compared to 1 μmol/L for diclofenac).

Our data support the hypothesis that increased circulating concentrations of albumin modified by Amadori glucose adducts plays an important role in the development of diabetic renal disease. Treatment with test compound, which normalized serum glycated albumin, arrested structural, functional and cell biology abnormalities associated with diabetic nephropathy even though the animals remained markedly hyperglycemic. Thus, inhibiting the formation of glycated albumin protected against the nephropathogenic effects of this glycated protein independent of anti-hyperglycemic therapy. It is un-

likely that inhibition of glycation of other serum proteins was responsible for the observed in vivo responses, since about 99% of this structural class of compounds binds to serum albumin [26] and the preponderance of in vitro [6, 7, 9] and in vivo [18–20] evidence indicates that Amadori-modified albumin is the main circulating glycated protein participatory in the pathogenesis of diabetic nephropathy.

Renal failure remains a common and serious problem in patients with type 1 or type 2 diabetes. The traditional approach to forestalling the onset or progression of diabetic nephropathy has focused on optimizing the control of blood glucose, reducing systemic factors and, more recently, modulating hemodynamic influences [1, 43–46]. These measures are not completely effective and, along with the recognition that strict glycemic control is difficult to achieve and may be associated with hypoglycemic episodes of unacceptable frequency or severity, is an appreciation that diabetic patients may require treatments specifically targeted at the vascular complications of this disease [47]. The inextricable links between glycated albumin-induced stimulation of glomerular TGF-β1 expression, increased extracellular matrix production and glomerular pathobiology, and the amelioration of these abnormalities in the face of persistent hyperglycemia by inhibiting the nonenzymatic glycation of albumin, suggest that this approach represents a useful therapeutic strategy for preventing the progression of diabetic nephropathy.

ACKNOWLEDGMENTS

This study was supported in part by grants EY11825, DK54143 and DK54608 from the National Institutes of Health, and by Exocell, Inc.

Reprint requests to Margo P. Cohen, M.D., Institute of Metabolic Research, University City Science Center, 3508 Market Street, Suite 420, Philadelphia, Pennsylvania 19104, USA.
E-mail: drmpcohen@aol.com

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